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Comparative electrophoretic investigation on *Rana balcanica* and *Rana ridibunda* from northern Greece

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A total of 315 water frogs pertaining to either *Rana ridibunda* or *Rana balcanica* was collected at 28 sites distributed over northern Greece. The intra- and interspecific variability of the gene pools was assessed by applying vertical polyacrylamide gel electrophoresis on samples of blood, liver, skeletal and heart muscle. We compared the allelic variation of eight enzyme loci and of two non-enzymatic proteins among populations and species using the UPGMA-, Fitch-Margoliash- and maximum-likelihood-methods. The variability between conspecific populations was considerably smaller than the interspecific variability. Moreover, at the ADA locus alternatively fixed alleles were found in *R. balcanica* and *R. ridibunda*. Thus, the species status of the bioacoustically detected *R. balcanica* was confirmed.

INTRODUCTION

Until recently *Rana ridibunda* Pallas, 1771 has been considered a monotypic species with a wide geographical range including northern Africa, Europe and the western regions of Asia (MERTENS & WERMUTH, 1960; GÜNTHER, 1990). However, intensive bioacoustic studies on features of the mating calls recorded in populations in Armenia (SCHNEIDER & EGIASARJAN, 1990), Egypt (AKEF & SCHNEIDER, 1989), Greece (SCHNEIDER & SOFIANIDOU, 1985; SCHNEIDER et al., 1984), Israel (NEVO & SCHNEIDER, 1983), Kazakhstan (SCHNEIDER & EGIASARJAN, 1991) and Turkey (JOERMANN et al., 1988) led to the unequivocal distinction of three different species within this geographical range (SCHNEIDER & SINSCH, 1992): *Rana ridibunda* in the terra typica restricta in Kazakhstan, and in Armenia, eastern Greece and Bulgaria, *Rana levantina* (SCHNEIDER et al., 1992) in Egypt, Israel and western

Turkey, and *Rana balcanica* (SCHNEIDER et al., 1993) in Greece, Albania and Yugoslavia. Additional morphometric comparisons revealed slight morphological differentiations between these species (SCHNEIDER et al., 1992, 1993). Analyses of allozyme variation among the bioacoustically detected taxa corroborated their species status (NEVO & FILIPUCCI, 1988; NEVO & YANG, 1982; SINSCH & EBLENKAMP, 1994; SOFIANIDOU, unpubl. data).

All previous studies on the allozyme variation between the new species *R. balcanica* and *R. ridibunda* have been performed on a rather large geographical scale and focused on resolving the taxonomic problems by comparison of bioacoustically classified populations. In the present investigation, allozyme electrophoresis was used to specify more precisely the distribution of *R. balcanica* and *R. ridibunda* in Greece. Our aims were: (1) to assess the genetic variability of each species on a small geographical scale by collecting samples along an east-west transect through northern Greece; (2) to compare the intraspecific and interspecific genetic variation; (3) to investigate the stability of genotypes at the neighbouring limits of geographical distribution at the Nestos River; and (4) to compare the results with those of the bioacoustic studies.

MATERIAL AND METHODS

At 28 sites distributed over northern Greece (Table I, fig. 1), we collected a total of 315 individuals of *Rana balcanica* and *Rana ridibunda* during three successive breeding periods. At the Epeiros sites where *R. balcanica* occurs syntopically with *R. epeirotica*, species identification was based on the mating calls. Specimens were measured, sexed and numbered. Samples of blood (plasma and red cell hemolysates), liver, skeletal and heart muscle were obtained from each specimen and stored at −35°C until electrophoretic analysis. The carcasses remaining after tissue sampling were preserved and deposited in the collection of the Laboratory of Zoology at the University of Thessaloniki.

For electrophoresis, we employed the vertical polyacrylamide gel technique using the SE 600 Hoefer apparatus. Gels included a stacking and a separating portion and were 1.5 mm or 0.75 mm thick. Electrophoresis was conducted under refrigeration (4-6°C). Mostly, a discontinuous buffer system was used (stacking gel buffer Tris-HCl, pH 6.8; resolving buffer Tris-HCl, pH 8.8; reservoir buffer Tris-glycine, pH 8.3), whereas in case of the LDH we employed a borate pH 8.2 system (PASTEUR et al., 1988). The samples (plasma and aqueous extracts) of tissues were run in gels with two different percentages of monomer in the separating gel (7.5 % T − 10 % T). The power supply was set for constant amperage (30 mA), and the duration of electrophoresis varied from 3-6 h depending on the protein.

Enzymes and other proteins were stained using standard procedures (PASTEUR et al., 1988). Proteins of different individuals were compared side by side on the same gel to avoid errors due to the slightly varying absolute mobilities in different gels. Enzyme systems examined were: adenosine deaminase (ADA, EC 3.5.4.4), α-glycerophosphate dehydrogenase (α-GPD, EC 1.1.1.8), lactate dehydrogenase (LDH, EC 1.1.1.27), malate

Table I. - List of sampling localities in Northern Greece and number of water frogs collected for protein analyses. The spatial relations between the sites are given in fig. 1.

	Locality	Number of individuals per sample		
		a	b	c
	Epeiros			
1.	Panvotis Lake (a and b at the same locality)	20	10	
2.	Louros River	10		
3.	Parakalamos (upper part of the Kalamas River)	6		
4.	Sagiada Wetland	20		
5.	Nea Selefkeia marsh	6		
6.	Kalodiki, swampy lake	10		
	Macedonia			
7.	Axios River, river side pond	20		
8.	Axios River, river bank	6		
9.	Doirani Lake	4		
10.	Gallikos River, estuary region	20		
11.	Gallikos River, 35 km north of site 10	6		
12.	Thessaloniki, brooklet in the forest Seih-Su	4		
13.	Lankadas, small river	10		
14.	Kerkini Lake	6		
15.	Agion Oros, Moni Zographu	3		
16.	Thasos Island	5		
17.	Nestos River, river bank	12		
18.	Nestos River, river side pond	6		
	Thrace			
19.	Kompsatos River	4		
20.	Thermes of Echinos	3		
21.	Vistonis Lake	20		
22.	Komotini, small river	13		
23.	Evros Delta	8		
24.	Samothraki Island	6		
25.	Erythropotamos River (near Didymoteicho)	8		
26.	Kufovuno, small branch of Erythropotamos	8		
27.	Valtos, Orestiada, brook (a and b at the same locality)	16	5	
28.	Ardas River, at Komara (a, b and c at the same locality)	22	7	9
	Total	284	22	9

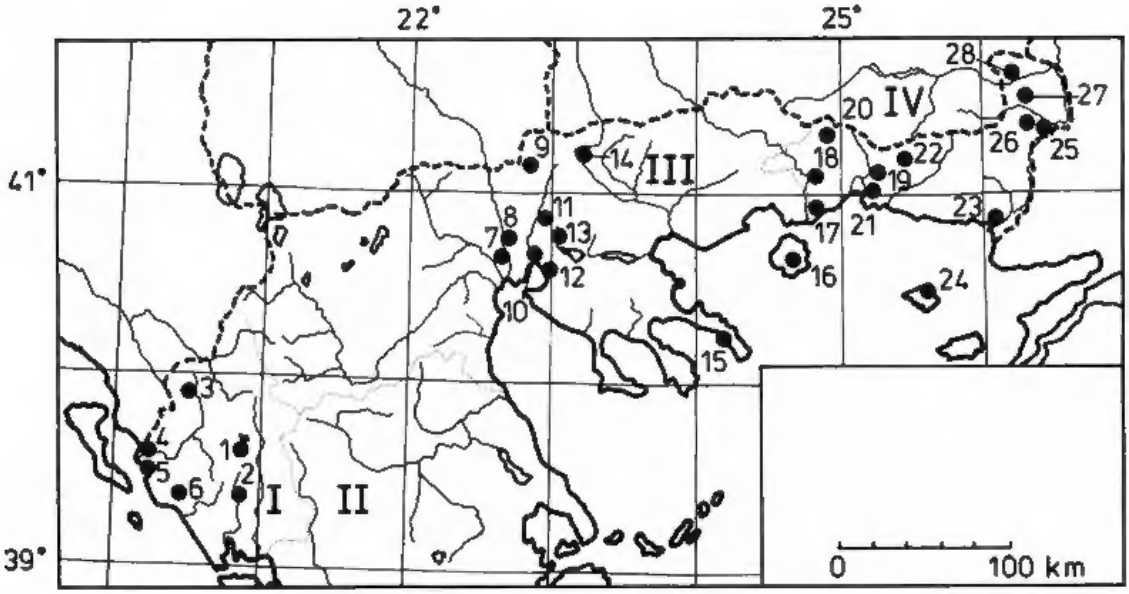

Fig. 1. – Map of northern Greece indicating the spatial relations between the sampling sites (dots and numbers) of water frogs. Table I gives the names of the sampling sites and the number of individuals collected. I: Epeirus; II: Thessalia; III: Macedonia; IV: Thrace.

dehydrogenase (MDH, EC 1.1.1.37), mannose phosphate isomerase (MPI, EC 5.3.1.8), 6-phosphogluconate dehydrogenase (6-PGD, EC 1.1.1. 37), phosphoglucomutase (PGM, EC 2.7.5.1). Additionally, the electromorphs of plasma albumin (ALB) and of a soluble muscle protein (MProt) were analyzed.

Several other loci such as MDH-2 or MProt-2 and non-enzymatic muscle and plasma proteins were scored in preliminary analyses and proved to be monomorphic. Since this study focused on taxonomic distinction between the two species and not on an estimate of their phylogenetic distance, we refrained from including the presumptive monomorphic loci into the main study.

Multiple loci were numbered according to the mobility of their products from anode to cathode. Stainable bands corresponding to the alleles of one presumptive locus were assigned letters according to their mobility, beginning with the band closest to the anode. Average heterozygosity per locus (H_o = observed frequency; H_e = expected frequency), proportion of polymorphic loci (P %), and the mean number of alleles per locus (A) were calculated for each sample. We used the G-test to detect deviations of the observed heterozygosity from the Hardy-Weinberg equilibrium, and the non-parametric Wilcoxon-Mann-Whitney U test (two-tailed) for the interspecific comparisons of H_o and P %. CAVALLI-SFORZA's chord distance (CAVALLI-SFORZA & EDWARDS, 1967) was calculated for all pairwise combinations of samples using the program GENDIST 3.4 of the package PHYLIP, version 3.4 (FELSENSTEIN, 1985). Although we did not consider presumably monomorphic loci, these estimates of genetic distance are useful to resolve the relative

genetic relationships between the examined populations by computing rooted and unrooted trees based on four algorithms: (1) UPGMA method (program NEIGHBOR 3.41); (2) FITCH-MARGOLIASH method assuming equal rates of evolutionary change in all lineages (KITSCH 3.41); (3) FITCH-MARGOLIASH method without evolutionary clock (FITCH 3.41); (4) maximum-likelihood method (CONTML 4.42). All calculations are based on the cited programs of the package PHYLIP (FELSENSTEIN, 1985).

RESULTS

ALLELIC VARIATIONS OF PROTEINS

A total of 10 presumptive loci (enzymes: ADA, α-GPD, LDH-1, LDH-2, MDH-1, MPI, 6-PGD, PGM-2; non-enzymatic proteins: ALB, MProt) were scored in 32 samples of frogs from 28 sites (Table II). Except for one (MDH-1), all loci were polymorphic, producing two to four bands of distinct electrophoretic mobility which we consider the results of the activity of different alleles of the same locus. The corresponding frequencies of the alleles are listed in Table II. Observed heterozygosity did not deviate significantly from expectations (H_e) in all but two populations (Vistonis Lake and Komotini River, significant deficit of heterozygotes). However, the average observed heterozygosity H_o ($P = 0.00388$, Wilcoxon U test), the proportion of polymorphic loci P % ($P = 0.000256$, U test), and the mean number of alleles per locus A ($P = 0.01723$, U test) were significantly greater in *R. ridibunda* populations than in *R. balcanica* populations.

The following account of the polymorphic loci demonstrates that (1) alternatively fixed alleles at the ADA locus permitted an unequivocal distinction between *R. balcanica* and *R. ridibunda* populations, and (2) there was a clear geographical variation of allele frequencies at other loci, which distinguished the *R. balcanica* populations of Epeiros from their conspecifics in Macedonia. We refrained from speculations on presumptive homologies of electromorphs detected in the present study with those in studies of other authors because different electrophoretic conditions render such comparisons unreliable.

Allozymes

Adenosine deaminase

The two alleles permitted an unequivocal electrophoretic discrimination between samples of *R. balcanica* from Epeiros and Macedonia and *R. ridibunda* from Thrace. *Rana balcanica* populations were fixed for the a allele, *R. ridibunda* populations for the b allele.

α-glycerophosphate dehydrogenase

All samples collected from *R. balcanica* populations were monomorphic for the a allele. The only exception was one of the two samples from the Nestos River, in which we also detected the b allele at a frequency of 25 %. In contrast, the b allele predominated in the *R. ridibunda* populations.

Table II. - Allele frequencies at ten presumptive loci in samples of water frogs from 25 localities through northern Greece. Numbers refer to the sites listed in Table I. Replicate samples are identified by, e.g., 1a and 1b for site 1. P %: relative frequency of polymorphic loci; A: average number of alleles per locus; H_e: relative frequency of expected heterozygosity; H_o: relative frequency of observed heterozygosity.

		1a	1b	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ADA	a	1.00	1.00	1.00	1.00	1.00	1.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-GPD	a	1.00	1.00	1.00	1.00	1.00	1.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LDH-1	a	1.00	1.00	1.00	1.00	1.00	1.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.33
LDH-2	a	1.00	1.00	1.00	1.00	1.00	1.0	1.00	1.00	1.00	1.00	1.00	-	1.00	1.00	1.00	1.00
	b	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-	-	-
MDH-1		1.00	1.00	1.00	1.00	1.00	1.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MPI	a	0.70	0.70	0.80	0.83	0.70	0.7	0.65	0.95	1.00	1.00	0.90	0.92	1.00	0.95	0.92	1.00
	b	0.30	0.30	0.20	0.17	0.30	0.3	0.35	0.05	-	-	0.10	0.08	-	0.05	0.08	-
6-PGD	a	-	-	-	-	-	-	-	0.05	0.08	-	0.10	-	-	0.05	0.08	-
	b	1.00	1.00	1.00	1.00	1.00	1.0	1.00	0.90	0.92	1.00	0.85	1.00	1.00	0.95	0.92	1.00
	c	-	-	-	-	-	-	-	0.05	-	-	0.05	-	-	-	-	-
PGM-2	a	0.35	0.40	0.30	0.33	0.30	0.17	0.05	0.10	-	-	0.05	-	-	0.05	0.08	-
	b	0.65	0.60	0.70	0.67	0.70	0.83	0.95	0.90	1.00	1.00	0.95	1.00	1.00	0.95	0.92	1.00
ALB	a	-	-	-	-	0.10	-	0.10	0.03	-	-	-	-	-	0.08	-	
	b	0.85	0.90	0.70	0.67	0.60	0.67	0.60	0.47	0.50	0.50	0.40	0.40	0.50	0.40	0.42	0.50
	c	0.15	0.10	0.30	0.33	0.30	0.33	0.30	0.50	0.50	0.50	0.43	0.60	0.50	0.60	0.50	0.50
	d	-	-	-	-	-	-	-	-	-	0.17	-	-	-	-	-	
MProt	a	-	-	-	-	-	-	-	0.03	-	-	-	-	-	-	-	-
	b	1.00	1.00	1.00	1.00	1.00	1.0	1.00	0.60	0.67	0.62	0.63	0.65	0.50	0.60	0.50	0.50
	c	-	-	-	-	-	-	-	0.37	0.33	0.38	0.37	0.35	0.50	0.40	0.50	0.50
P %		0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.5	0.3	0.2	0.5	0.3	0.2	0.5	0.5	0.3
A		1.3	1.3	1.3	1.3	1.4	1.3	1.4	1.8	1.3	1.2	1.7	1.3	1.2	1.5	1.6	1.3
H_e		0.11	0.11	0.12	0.12	0.14	0.11	0.11	0.15	0.10	0.10	0.16	0.11	0.10	0.12	0.15	0.14
H_o		0.11	0.10	0.11	0.10	0.13	0.08	0.10	0.11	0.10	0.10	0.16	0.10	0.10	0.11	0.11	0.15

Table II. - Continuation.

		16	17	18	19	20	21	22	23	24	25	26	27a	27b	28a	28b	28c
ADA	a	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-		
	b	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
α-GPD	a	1.00	0.75	1.00	0.25	0.33	0.40	0.29	0.38	0.50	0.25	0.30	0.30	0.20	0.27	0.34	0.50
	b	-	0.25	-	0.75	0.67	0.60	0.71	0.62	0.50	0.75	0.70	0.70	0.80	0.73	0.66	0.50
LDH-1	a	1.00	0.83	0.67	-	-	0.45	0.31	0.13	0.50	-	-	0.10	-	0.27	0.43	0.44
	b	-	-	0.33	0.25	1.00	-	-	-	-	-	-	-	-	-	-	0.17
	c	-	0.17	-	0.75	-	0.55	0.69	0.87	0.50	1.00	1.00	0.90	1.00	0.73	0.57	0.39
LDH-2	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MDH-1		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MPI	a	0.90	0.60	0.50	0.50	0.33	0.25	0.31	0.25	0.50	0.25	0.30	0.30		0.27		
	b	0.10	0.40	0.50	0.50	0.67	0.75	0.69	0.75	0.50	0.75	0.70	0.70		0.73		
6-PGD	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	1.00	0.83	0.87	-	-	-	-	-	-	-	-	-	-	0.14	0.33	-
	c	-	0.17	0.13	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.86	0.67	1.00
PGM-2	a	-	0.08	-	0.62	0.67	0.52	0.69	0.69	0.67	0.75	0.75	0.70	0.80	0.75		
	b	1.00	0.92	1.00	0.38	0.33	0.48	0.31	0.31	0.33	0.25	0.25	0.30	0.20	0.25		
ALB	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	0.40	0.43	0.50	0.38	0.50	0.35	0.37	0.38	0.33	0.50	0.30	0.34	0.30	0.39	-	0.30
	c	0.60	0.54	0.50	0.62	0.50	0.65	0.63	0.62	0.67	0.50	0.70	0.63	0.70	0.61	0.67	0.40
	d	-	0.03	-	-	-	-	-	-	-	-	-	0.03	-	-	0.33	0.20
MProt	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	b	0.60	0.25	0.13	-	-	0.10	-	-	0.17	-	-	-	-	0.05	-	
	c	0.40	0.75	0.87	1.00	1.00	0.90	1.00	1.00	0.83	1.00	1.00	1.00	1.00	0.95	1.00	
P %		0.3	0.7	0.5	0.5	0.4	0.6	0.5	0.5	0.6	0.5	0.4	0.5		0.7		
A		1.3	1.8	1.5	1.5	1.4	1.6	1.5	1.5	1.6	1.4	1.4	1.6		1.7		
H_e		0.11	0.22	0.19	0.22	0.18	0.27	0.22	0.20	0.21	0.16	0.16	0.20		0.26		
H_o		0.10	0.22	0.17	0.15	0.20	0.19	0.10	0.13	0.20	0.13	0.15	0.17		0.20		

Lactate dehydrogenase

Three alleles were detected at the LDH-1 locus. However, in 16 of the 18 *R. balcanica* populations this locus was monomorphic for the a allele. Only at Agion Oros did the c allele, and at the Nestos River the b and c alleles, also occur. In contrast, the c allele dominated over the a and b alleles in all *R. ridibunda* populations, except of (1) Samothraki where all 6 individuals were heterozygous (a/c) and of (2) Komara (replicate sample 28c) where the a allele dominated. Heterozygotes of the a/c constitution (6 at Samothraki, 2 at Nestos River, 2 at Lake Vistonis, 1 at Valtos, 5 at Komara) showed the

typical five-banded pattern, whereas, due to the small difference in electrophoretic mobility of a and b gene products, a/b heterozygotes (1 at Nestos River, 1 at Komara) showed only two readily distinguishable bands.

The LDH-2 locus was monomorphic in all but one populations. The extremely rare b allele was detected during a repeated sampling in six individuals from the Gallikos River.

Mannose phosphate isomerase

There were conspicuous geographical differences in the frequencies of the two detected alleles at this locus. The *R. balcanica* populations of Macedonia were almost monomorphic for the a allele, whereas the frequency of the b allele increased to about 30 % in the conspecific populations of Epeiros. In the *R. ridibunda* populations, the frequency of the b allele ranged between 50 and 76 %. Notable exceptions to this general pattern were the *R. balcanica* samples from the Nestos River, with allele frequencies resembling those of *R. ridibunda* populations.

6-phosphogluconate dehydrogenase

The three alleles detected at the 6-PGD locus again showed a clear geographical segregation. The *R. balcanica* populations of Epeiros were monomorphic for the b allele, whereas low frequencies of the a and c alleles were present in the conspecific populations of Macedonia besides the dominant b allele. In contrast, the *R. ridibunda* populations of Thrace were monomorphic for the c allele (except for Komara: low frequencies of the b allele).

Phosphoglucomutase

As in the allelic distribution in the MPI, the frequencies of the two alleles at the PGM-2 locus varied geographically. The *R. balcanica* populations of Macedonia were almost monomorphic for the b allele, whereas the frequency of the a allele increased to about 30 % in the conspecific populations of Epeiros. In the *R. ridibunda* populations, the frequency of the a allele increased even more, to about 70 %. The a/b heterozygotes showed a broad band ranging from the positions of the homozygote a and b bands.

Non-enzymatic proteins

Albumin

The frequencies of the most common alleles, b and c, varied clinally along an east-west axis in *R. balcanica* and in *R. ridibunda*. Allele d was frequent only at Komara and at Gallikos River, whereas it was rare at Nestos River and at Valtos and absent in all other samples. Allele a occurred in low frequencies at some localities in Epeiros and Macedonia, i.e. exclusively in populations of *R. balcanica*.

Muscle protein

The three different stainable bands found in the fraction of soluble muscle protein showed geographically and interspecifically varying frequencies. In the *R. balcanica*

populations of Epeiros, only the b band was detected, whereas in the populations of Macedonia the c band reached frequencies ranging from 33 to 87 %. The rare a band was found only once, at the Axios River. Finally, the c band was often the only one detected in the *R. ridibunda* populations, but at some sites low frequencies of the b bands were also found. Frequent two-banded heterozygotes of the b/c type indicated that the three bands are probably due to allelic variation of the same locus.

GENETIC DIFFERENTIATION

The genetic variation between samples collected at the same or nearby sites (< 1 km), and between samples from different localities, was assessed by calculating CAVALLI-SFORZA's chord distance (Table III). However, we excluded the replicate samples from Valtos and Komara (sites 27 and 28 in Thrace) because of missing data which might bias the comparison

Random effects of sampling

The chord distances between samples collected at the same site (Panvotis Lake), and between those collected in close vicinity (Axios River, Nestos River), ranged between 0.001 and 0.133. These three sites were inhabited by *R. balcanica* populations.

Intraspecific variation

The chord distances between the 18 *R. balcanica* populations ranged from 0.005 between the Louros and Kalamas Rivers to 0.462 between the geographically distant localities Panvotis Lake and Gallikos River. Within the major geographical regions Epeiros and Macedonia, genetic variation was considerably smaller. The six populations from Epeiros were genetically very similar, with chord distances between 0.005 and 0.056 (median: 0.018). The twelve populations from Macedonia were less uniform, with chord distances ranging between 0.012 and 0.438 (median: 0.119). Finally, the intraspecific genetic variation among the ten *R. ridibunda* populations from Thrace varied with chord distances of 0.006-0.324 (median: 0.072) within the range of the *R. balcanica* populations from Macedonia.

Interspecific variation

Finally, the comparison between the populations of *R. balcanica* and *R. ridibunda* yielded chord distances in the range of 0.553 to 1.625 (median: 1.197). Even the smallest chord distance between any interspecific pair was still greater than the greatest distance between pairs of conspecific populations

Grouping of populations

All populations examined were grouped with respect to their genetic similarity using four algorithms which are commonly applied in the reconstruction of phylogenetic relationships. All algorithms led to identical or similar groupings of populations.

Table III - CAVALLI-SFORZA's chord distances between the water frog populations of 25 sampling sites. The replicate samples 27b, 28b and 28c were excluded because not all loci were scored

	1a	1b	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27a	28a
1a	-	0.001	0.007	0.010	0.022	0.013	0.045	0.137	0.187	0.184	0.161	0.442	0.207	0.146	0.161	0.259	0.163	0.282	0.336	1.339	1.321	1.041	1.193	1.233	0.972	1.362	1.369	1.268	1.048
1b		-	0.013	0.016	0.028	0.022	0.056	0.151	0.205	0.202	0.175	0.462	0.225	0.163	0.176	0.277	0.183	0.298	0.354	1.346	1.326	1.051	1.200	1.240	0.980	1.365	1.376	1.275	1.053
2			-	0.005	0.017	0.006	0.037	0.108	0.146	0.144	0.134	0.406	0.167	0.111	0.132	0.218	0.127	0.265	0.321	1.331	1.325	1.039	1.192	1.235	0.962	1.370	1.365	1.267	1.050
3				-	0.018	0.008	0.042	0.106	0.145	0.142	0.134	0.406	0.165	0.109	0.131	0.217	0.128	0.268	0.328	1.329	1.326	1.040	1.191	1.235	0.959	1.370	1.364	1.266	1.049
4					-	0.018	0.018	0.114	0.175	0.173	0.152	0.424	0.196	0.132	0.126	0.247	0.145	0.271	0.325	1.333	1.327	1.032	1.188	1.229	0.964	1.366	1.360	1.262	1.045
5						-	0.021	0.108	0.140	0.137	0.127	0.389	0.160	0.106	0.128	0.212	0.110	0.248	0.290	1.337	1.329	1.032	1.194	1.235	0.970	1.374	1.370	1.269	1.054
6							-	0.112	0.145	0.142	0.138	0.390	0.165	0.120	0.119	0.217	0.110	0.259	0.283	1.379	1.372	1.065	1.237	1.277	1.014	1.420	1.415	1.312	1.099
7								-	0.039	0.045	0.038	0.325	0.047	0.018	0.017	0.099	0.040	0.139	0.168	1.145	1.161	0.888	1.022	1.069	0.819	1.216	1.196	1.097	0.916
8									-	0.012	0.055	0.311	0.016	0.017	0.040	0.068	0.029	0.207	0.214	1.317	1.344	1.061	1.207	1.257	0.988	1.407	1.386	1.284	1.099
9										-	0.070	0.299	0.021	0.023	0.049	0.054	0.016	0.191	0.195	1.305	1.332	1.052	1.195	1.245	0.981	1.395	1.374	1.272	1.085
10											-	0.342	0.072	0.037	0.047	0.124	0.056	0.133	0.174	1.169	1.182	0.902	1.042	1.088	0.841	1.237	1.217	1.097	0.939
11												-	0.302	0.301	0.325	0.354	0.286	0.438	0.438	1.547	1.566	1.276	1.425	1.472	1.218	1.625	1.601	1.500	1.311
12													-	0.025	0.047	0.052	0.018	0.180	0.180	1.279	1.306	1.035	1.169	1.219	0.967	1.369	1.348	1.246	1.065
13														-	0.015	0.076	0.016	0.149	0.168	1.212	1.232	0.953	1.090	1.138	0.887	1.289	1.264	1.165	0.979
14															-	0.099	0.037	0.148	0.165	1.184	1.199	0.930	1.058	1.105	0.868	1.253	1.232	1.133	0.952
15																-	0.069	0.159	0.222	1.137	1.306	0.948	1.062	1.085	0.887	1.205	1.184	1.106	0.951
16																	-	0.143	0.140	1.245	1.263	0.975	1.121	1.167	0.921	1.321	1.298	1.197	1.010
17																		-	0.133	0.773	0.882	0.564	0.650	0.684	0.553	0.800	0.790	0.697	0.562
18																			-	0.946	0.857	0.803	0.910	0.937	0.799	1.069	1.058	0.971	0.833
19																				-	0.151	0.132	0.087	0.068	0.147	0.053	0.049	0.063	0.113
20																					-	0.309	0.289	0.289	0.324	0.290	0.293	0.294	0.318
21																						-	0.025	0.038	0.017	0.104	0.099	0.050	0.039
22																							-	0.009	0.043	0.052	0.050	0.015	0.029
23																								-	0.062	0.025	0.022	0.006	0.035
24																									-	0.134	0.122	0.072	0.053
25																										-	0.007	0.024	0.072
26																											-	0.020	0.071
27a																												-	0.041
28a																													-

Therefore, we present the unrooted FITCH-MARGOLIASH-tree (fig. 2) as an example of the three algorithms based on CAVALLI-SFORZA's chord distance matrix and the unrooted maximum-likelihood-tree (fig. 3) directly calculated from the allele frequencies. The best FITCH-MARGOLIASH dendrogram with contemporary tips out of 4394 examined had a sum of squares of 50.023 with a corresponding average percent standard deviation of 24 851 The best unrooted FITCH-MARGOLIASH tree out of 7093 examined showed a better fit (sum of squares: 14 611; average percent standard deviation: 13.431). The best unrooted maximum-likelihood tree out of 1931 examined had a ln likelihood of 760.407.

Within the *R. balcanica* populations, there were two major clusters of populations corresponding to the samples from Epeiros (sites 1-6) and to those from Macedonia (7-16). Despite the occurrence of the unique LDH-2 allele b in a sample from the Gallikos River (site 11), this sample was included in the Macedonia cluster in the two unrooted trees (figs. 2-3), whereas the two rooted dendrograms (UPGMA, FITCH-MARGOLIASH with contemporary tips) failed to recognize this association and placed this population as an outgroup of all other *R. balcanica* populations. All reconstructions coincided in assigning the population from the Nestos River a position apart from the main clusters, but clearly within the branch of *R. balcanica*. Nevertheless, Balkan frogs from the Nestos River differed from those of all other sites by the introgression of *R. ridibunda* genome (e.g. α-GPD, LDH-1, MProt, 6-PGD).

Eight out of ten *R. ridibunda* populations (sites 21-28) joined one major cluster similar to those representing *R. balcanica* in Epeiros and Macedonia. However, again two populations (sites 19-20: Kompsatos River and Thermes of Echinos) differed considerably from all other conspecifics due to the presence of a rare allele of the LDH-1.

DISCUSSION

The allelic variation of proteins among 28 populations of water frogs in northern Greece corroborates the previous bioacoustic finding that *R. balcanica* inhabits the region west of the Nestos River and *R. ridibunda* the eastern region (SCHNEIDER & SINSCH, 1992). The populations at sites 1-18 (Table I) correspond to *R. balcanica*, those at sites 19-28 to *R. ridibunda*. The alternative fixation of the ADA alleles permitted an unequivocal biochemical distinction of the two species under these electrophoretic conditions

As we deliberately selected the polymorphic loci for our purpose of taxonomic distinction, and did not follow the presumably monomorphic loci detected, we chose CAVALLI-SFORZA's chord distance instead of NEI's (1972) genetic distance as a measure of genetic differentiation. The calculation of NEI's genetic distances from our data set would have led to an overestimation of the real genetic differentiation due to restriction on polymorphic loci. Therefore, our study presents relative values of genetic differentiation among the populations studied which cannot be compared directly with NEI's distances published elsewhere. However, CAVALLI-SFORZA's chord distance is a useful measure to decide whether the genetic differentiation between *R. balcanica* and *R ridibunda* supports the proposed species status or not.

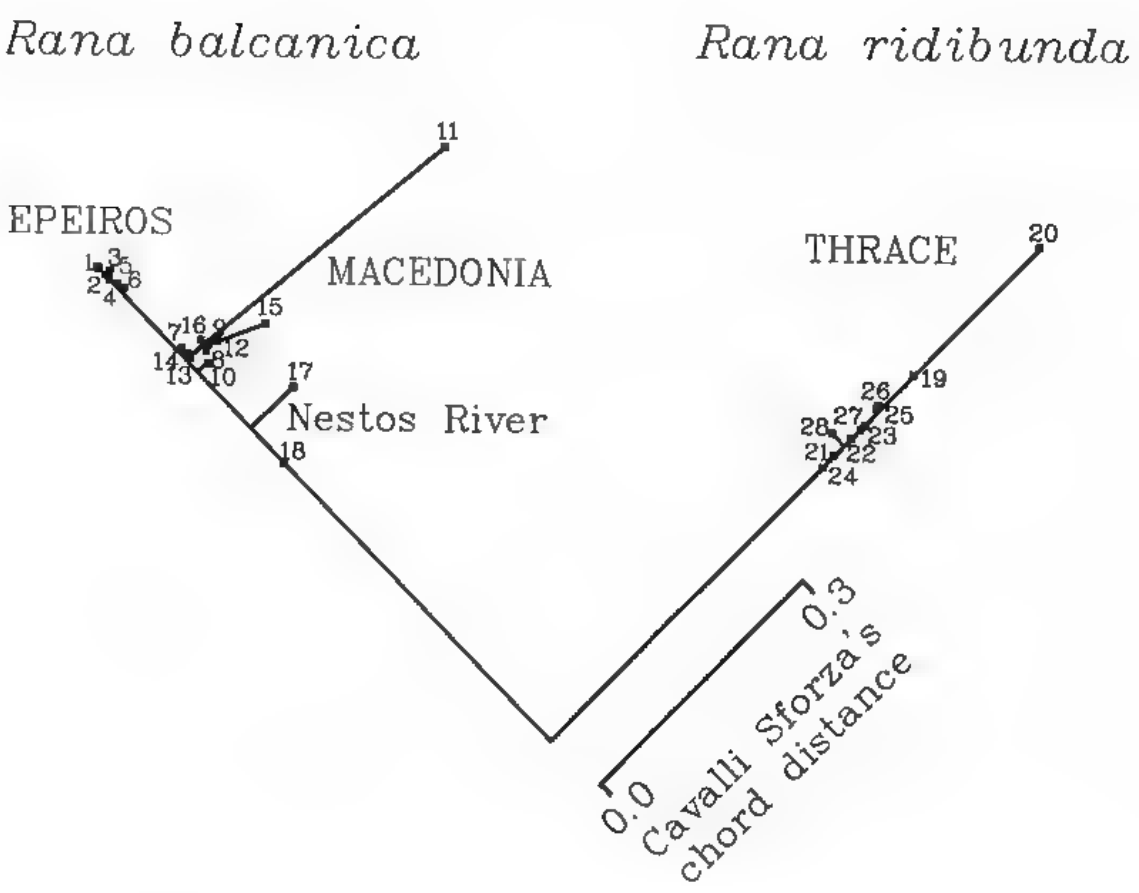

Fig. 2. – FITCH-MARGOLIASH tree (best one out of 7093 examined, sum of squares: 14.61, average percent standard deviation: 13.43) based on CAVALLI-SFORZA's chord distance matrix (Table III) of all pairwise combinations of the samples. Due to the absence of an outgroup this tree is arbitrarily rooted at the mean distance between the most similar populations of *R. balcanica* and *R. ridibunda*. The localities corresponding to the numbers of the populations are listed in Table I

INTRASPECIFIC GENETIC DIFFERENTIATION

The chord distances separating conspecific populations within a given region (Epeiros, Macedonia, Thrace) are low, but increase with geographical distance as should be expected. HOTZ & UZZELL (1982) also found low NEI's distances (D = 0.00-0.02) in water frogs of southwestern Greece (now classified as *R. balcanica*), and NEVO & YANG (1982) in water frogs of Israel (now classified as *R. levantina*; D = 0.006-0.056). This genetic homogeneity is probably due to the extensive exchange of individuals among neighbouring populations. This is not surprising as *R. balcanica* is known to migrate over large distances (SOFIANIDOU & SCHNEIDER, 1989), as do other European water frogs (15 km within one activity period in adult *R. lessonae* and *R. esculenta*; TUNNER, 1992), and juveniles usually disperse over even larger distances than adults (SINSCH, 1991, 1992).

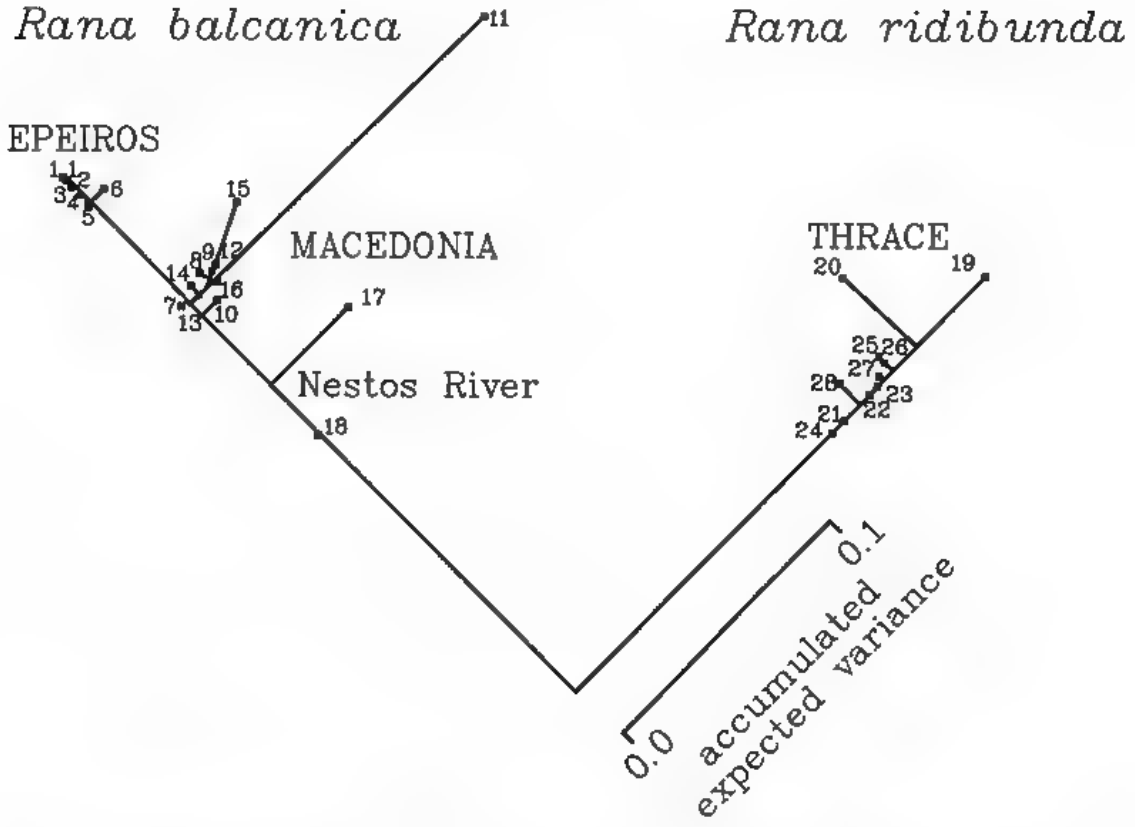

Fig. 3. – Maximum-likelihood tree (best one out of 1931 examined with ln likelihood: 760.407) based on the allele frequencies (Table II) of each sample. Due to the absence of an outgroup this tree is arbitrarily rooted at the mean distance between the most similar populations of *R. balcanica* and *R. ridibunda* The localities corresponding to the numbers of the populations are listed in Table I.

Nevertheless, we detected two notable exceptions to the general pattern, the *R. ridibunda* populations at the Kompsatos River (site 19) and Thermes of Echinos (site 20), and the *R balcanica* populations at the Nestos River (site 17-18) These four populations conspicuously deviated from the other conspecific ones, but still were clearly assignable to their respective species because of the alternatively fixed alleles of the ADA-locus. The *R. ridibunda* from the Kompsatos River and Thermes of Echinos differed from all others in the frequency of the b allele at the LDH-1 locus. Both localities are situated in the highlands, and altitude associated with warm water at the Thermes of Echinos may represent a selective pressure in favour of this rare allele.

At the Nestos River, the situation is more complicated. We know from bioacoustic and allozyme studies (SCHNEIDER et al., 1993, SINSCH & EBLENKAMP, 1994) that *R. balcanica* and *R. ridibunda* occur syntopically in this region. SCHNEIDER et al. (1993) detected a considerable character displacement in several mating call parameters which maximize the differences of the mating call between the two species. SINSCH & EBLENKAMP (1994), in turn, detected specific differences in the genotypes of several enzymes in *R.*

ridibunda of the Nestos region as compared to the genotypes from the brook at Valtos. This study has revealed the introgression of *R. ridibunda* genome into the *R. balcanica* genome at the Nestos River, e.g. allele b of the α-GPD and alleles b and c of the LDH-1. The alternatively fixed alleles of the ADA locus show that the occurrence of typical *R. ridibunda* alleles at other loci is due to introgression and not to an erroneous classification of some individuals of the Nestos samples. All these findings emphasize the importance of a further thorough study of the water frog populations in this area of distributional overlap between the two species.

Interspecific genetic differentiation

Our study clearly demonstrates that the chord distances between conspecific populations, even if they are separated by a large geographical distance, are always smaller than those between any interspecific pair. This genetic divergence holds also in the contact zone in the region around the Nestos River which apparently represents an ancient hybrid zone.

There are few estimates of Nei's distances between the three species of water frogs which were formerly referred to as *R. ridibunda* because they have only recently been distinguished: (1) *R. ridibunda* – *R. balcanica*: D = 0.082 (Sinsch & Eblenkamp, 1994); (2) *R. ridibunda* – *R. levantina*: D = 0.178 (Sinsch & Eblenkamp, 1994); (3) *R. balcanica* – *R. levantina*: D = 0.247 (Nevo & Filippucci, 1988) and D = 0.196 (Sinsch & Eblenkamp, 1994). All estimates of the genetic distances between the three species clearly fall into the normal range of genetic differentiation at species level in Amphibia (D = 0.1 – 3.0; Avise & Aquadro, 1982).

Protein analyses and bioacoustics

This investigation of the enzymatic and non-enzymatic proteins of the water frogs in northern Greece provides excellent corroboration of the results of previous bioacoustic analyses (Schneider & Sofianidou, 1985, 1986; Schneider & Sinsch, 1992; Schneider et al., 1984, 1993). It confirms both the presence of the two species *R. ridibunda* and *R. balcanica* and the local differences within *R. balcanica* (see figs. 2-3). The mating calls of *R. balcanica* in Epeiros have on average fewer pulse groups per call, and longer intervals between the pulses groups, than those of *R. balcanica* in Macedonia (Schneider & Sofianidou, 1986). But despite these local differences the mating calls of all populations are clearly classifiable as *R. balcanica* calls (Schneider et al., 1993).

In view of these protein analysis results, it seems desirable to continue both the bioacoustic and the electrophoretic studies in Greece, especially in the vicinity of the Nestos River and adjacent regions in Thrace and Macedonia, in order to determine where *R. ridibunda* and *R. balcanica* are sympatric and whether hybrids of the two species are present there. Hybrids have been found in other regions where two water frog species are sympatric: for example, hybrids of *R. ridibunda* and *R. lessonae* (Berger, 1964, 1973), of *R. ridibunda* and *R. perezi* (Graf et al., 1977), and of *R. balcanica* and *R. epeirotica*

(SOFIANIDOU & SCHNEIDER, 1987). Therefore, it seems rather likely that hybrids of *R. ridibunda* and *R. balcanica* will eventually be found.

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